The Seoul Journal of Medicine
Vol. 33, No. 3: 213-218, September 1992

Identification of *Mycobacterium tuberculosis* by Dot Blot Hybridization Using Digoxigenin-labeled Probe

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= Abstract =**_Mycobacterium tuberculosis_ H37Rv ATCC 27294, restricted with EcoRI, was used to construct a genomic library in a bacteriophage expression vector, Lambda gt11. One 1.5-kb DNA fragment was selected by picoBlue immunoscreening kit on the basis of the ability to encode _M. bovis_-specific protein. Recombinant pBluescript with the 1.5-kb DNA was constructed into EcoRI site. Denatured DNA solution was blotted with BIO-DOT microfiltration apparatus(BIO-RAD) onto nylon membrane. The 1.5-kb probe, labeled with digoxigenin, reacted strongly with M. tuberculosis H37Rv, _M. bovis_, and 5 clinical isolates of _M. tuberculosis_. No hybridizations were observed with _M. tuberculosis_ H37Ra, _M. scrofulaceum_, _M. intracellulare_, _S. aureus_, _S. epidermidis_, _S. marcescens_, _K. pneumoniae_, _E. coli_, _E. cloacae_, _P. vulgaris_, and _P. aeruginosa_. When purified DNA from _M. tuberculosis_ H37Rv was spotted onto nylon membrane, the 1.5-kb probe was able to detect 100 ng of DNA, which corresponds to 2 X 10^7 mycobacteria. This dot blot hybridization method using digoxigenin-labeled 1.5-kb probe may be applicable for identifying _M. tuberculosis_ in hospital laboratories.**

Key Words: *Mycobacterium tuberculosis, Dot blot hybridization, Digoxigenin, Lambda gt11, Expression library*

INTRODUCTION

The growth rate for the mycobacteria is slow and requires 2 to 8 weeks or longer for detection by use of traditional media. Mycobacteria are usually identified by rate of growth, colonial morphology, pigmentation, and biochemical profiles. It takes an additional 2 to 6 weeks for mycobacterial identification to species level after mycobacterial colonies are isolated. BACTEC system, gas-liquid chromatography, and nucleic acid probe technique have been recently introduced for the rapid detection and identification of the mycobacteria (Roberts *et al.* 1991).

A cDNA probe system is commercially available from Gen-Probe for rapid diagnosis of tuberculosis. But this system is too expensive to be routinely used in most hospital laboratories.

This study intended to clone a DNA sequence unique to *M. tuberculosis* complex that could be hybridized to DNA from organisms for identification. A genomic library in bacteriophage λgtII was constructed with *M. tuberculosis* H37Rv strain, restricted with EcoRI, which was screened by polyclonal antibody to *M. bovis*. Chromosomal DNA was blotted with BIO-DOT apparatus onto nylon membrane and probe DNA was labeled with digoxigenin.

Received June 1992, and in final form September 1992.

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In the present report, the development and testing of nonisotope digoxigenin-labeled DNA probe specific for *M. tuberculosis* complex by dot-blot hybridization is described.

MATERIALS AND METHODS

Isolation of mycobacterial DNA:

M. tuberculosis H37Rv ATCC 27294 (90-03, MED. 502) was grown by inoculating 100 ml of Middlebrook 7H9 broth (DIFCO, 0713-01) supplemented with 0.05% Tween 80 and OADC enrichment (DIFCO, 0722-64-0). The culture was incubated with constant shaking of 60 rpm at 37℃ for 7 to 10 days. D-cycloserine (Sigma, C 6880) was then added to the final concentration, 1 mg/ml (Shoemaker *et al.* 1985).

1 to 2 days later the mycobacteria solution was heated at 70℃ for 20 min, and centrifuged at 3,000 rpm for 30 min (Collins and De Lisle 1984). The mycobacterial pellet was washed in 20 ml of STE buffer consisting of 0.01 M Tris-HCl, 0.1 M NaCl, and 0.001 M EDTA (pH 8.0). The pellet was resuspended in lysis solution consisting of 2 ml of 15% sucrose, 50 mM EDTA, and 50 mM Tris-HCl (pH 8.0), and 100 μl of lysozyme (Sigma, L6876; 10 mg/ml). After 2 hours at 37℃, 20 μl of proteinase K (Boehringer Mannheim, 745723; 20 mg/ml), 400 μl of 10 % sodium dodecyl sulfate (SDS), and 2.5 ml of distilled water were added.

After 18 hours at 37℃, 5 ml of phenol/chloroform/isoamyl alcohol (25:24:1) was mixed and centrifuged at 2,000 rpm for 5 min.

Chloroform/isoamyl alcohol (24:1) was added to the supernatant and centrifuged at 2,000 rpm for 5 min. 75 μl of ribonuclease A (Sigma, R9005; 10 mg/ml) was added to the supernatant and incubated at 37℃ for 2 hours.

The solution was dialyzed in 3,000 ml of 50 mM Tris-HCl and 1 mM EDTA (pH 8.0) at 4℃ for 16 to 18 hours (Eisenach *et al.* 1986).

Construction of DNA library in lambda gt11:

DNA from *M. tuberculosis* H37Rv ATCC 27294 was selected for library construction and was digested with the restriction endonuclease EcoRI (New England Biolabs).

1 μg of total EcoRI fragments was used for the ligation with λgt11 arms by using Lambda gt 11 cloning Kit (Stratagene, 234211). The recombinant phages were packaged by using the Gigapack II plus packaging extract kit (Stratagene, 200211) and absorbed to *Escherichia* coli Y1088. Library amplification was done by the method of Huynh *et al.* (1985).

Immunoscreening of recombinant λgt11:

Rabbit anti-*Mycobacterium bovis* polyclonal antiserum (DAKOPATTS, B124, Lot No. 063) and picoBlue immunoscreening kit (Stratagene, 200371) were used for the immunoscreening of recombinant λgt11. Final dilution of anti-*Mycobacterium bovis* was 1:3,000 into blocking solution (1 % bovine serum albumin in Tris-buffered saline, 7.5 ml/filter).

Isolation of recombinant λgt11 DNA:

One plaque of tertiary screening was eluted in 1 ml of SM buffer and 1-2 drops of chloroform at 4℃ for 2 hours (Ausubel *et al.* 1987). 0.1 ml of *E. coli* Y1088 were incubated in suspension with 0.1 ml of 10 mM $MgCl_2$ and 10 mM CaCl2 at 37℃ for 15 min. This mixture was added into 50 ml of lambda broth and incubated with strong shaking at 37℃ for 6 to 8 hours.

After adding 5 drops of chloroform, the solution was centrifuged with refrigerated Centrikon T-324 (KONTRON Inst., rotor A8.24) at 10,000 rpm for 10 min.

10 μl of DNase I (Boehringer Mannheim, 104159; 5 mg/ml) and 25 μl of RNase A (10 mg/ml) were added into the supernatant. After incubation at 37℃ for 1 hour, the solution was centrifuged at 20,000 rpm for 2 hours 15 min. The pellet was dissolved with 200 l of 0.05 M Tris-HCl, pH 8.0, DNA was purified by phenol-chloroform extraction and ethanol precipitation. Insert DNA was isolated from EcoRI-digested recombinant λgt11 DNA by Geneclean II kit (BIO 101 Inc.)

Subcloning *M. tuberculosis* DNA insert into a plasmid vector:

Insert DNA was ligated into plasmid vector pBluescript II phagemid kit (Stratagene, 212205). Recombinant plasmid was transformed into competent *E. coli* XLI-Blue according to the method of Sambrook *et al.* (1989). Amplified recombinant plasmid was isolated from *E. coli* XL1-blue by using Circleprep Kit (BIO 101 Inc). Insert DNA was isolated from EcoRI-digested recombinant plasmid by using Geneclean II Kit.

Dot blotting:

Mycobacterial DNA was blotted onto nylon membrane by using BIO-DOT microfiltration apparatus (B10-RAD). 25 μl (100 ng) of DNA and 25 μl of 0.8 M NaOH/20 mM EDTA were mixed and heated for 10 min on boiling water. 50 μl of cold 2 M ammonium acetate was added into the cooled DNA solution.

Total 100 μl solution was blotted onto nylon membrane soaked with 6 X SSC solution (20 X SSC; 3 M NaCl and 0.3 M sodium citrate, pH 7.0). After blotting, 50 μl of 2 X SSC solution was aspirated. The membrane was dried at 80℃ in a vacuum oven for 2 hours.

Digoxigenin labeling and hybridization:

Insert DNA was labeled with digoxigenin-dUTP and chromosomal DNA blotted onto nylon membrane was hybridized by using DNA labeling and Detection Kit Nonradioactive (Boehringer Mannheim 1093657).

Prehybridization was performed with 20 ml of hybridization solution (50% formamide, 5 X SSC, 5% blocking agent, 0.1% N-lauroyl sarcosine, 0.02% SDS) at 50 for 3 hours. Labeled DNA was added into 10 ml of new hybridization solution. Hybridization was performed at 50℃ overnight with 60-rpm shaking. Nylon membrane was washed with 2 X SSC/0.1 % SDS solution at 72℃ for 30 min, twice and washed again with 0.1 X SSC/0.1 % SDS solution at 72℃ for 2 hours.

Polyclonal sheep anti-digoxigenin Fab fragment conjugated to alkaline phosphatase, found to DNA probe, was visualized by nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate solution.

RESULTS

1.0—2.5 mg of mycobacterial chromosomal DNA could be obtained from the 100-ml culture of enriched Middlebrook 7H9 broth by the modified Eisenach method.

5—10 positive plaques were observed among about 3,000 plaque forming units per plate when primary screening was done with rabbit anti-*Mycobacterium bovis* antidody. After liquid lysate DNA of positive λgt11 plaque was digested with EcoRI, one 1.5-kb insert DNA was observed on the electrophoresis.

This 1.5-kb DNA fragment was ligated with pBluescript on EcoRI site and 4.4-kb recombinant plasmid could be obtained and named as pBSTB (Fig. 1).

When purified DNA from *M. tuberculosis* H37Rv was blotted onto nylon membrane in 100 ng, 10 ng, and 1 ng, this dot blot hybridization system with digoxigenin-labeled 1.5-Kb DNA

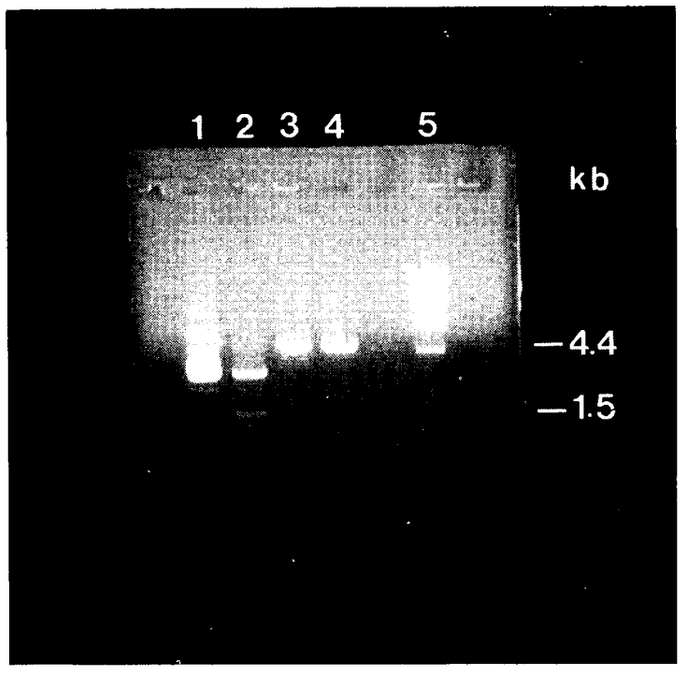

Fig. 1. Restriction endonuclease analysis of recombinant pBluescript. Lanes: 1, undigested; 2, EcoRI-digested; 3, HindIII-digested; 4, BamHI-digested; 5, HindIII-digested Lambda phage.

probe was able to detect 100 ng of DNA, which corresponds to 2 X 10^7 mycobacteria on the basis of an estimated genome molecular weight of 2.56 X 10^9 dalton (Baess 1984).

With DNA-DNA hybridization, the 1.5-Kb probe, labeled with digoxigenin, reacted strongly with *M. tuberculosis* H37Rv ATCC 27294, *M. bovis* ATCC 19210, and 5 clinical isolates of *M. tuberculosis* (Fig. 2).

No hybridizations of the 1.5-Kb DNA probe were observed with *M. tuberculosis* H37Ra ATCC 25177, *M. intracellulare* ATCC 13950, *M. scrofulaceum* ATCC 19981, *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228, *S. marcescens* ATCC 8100, *K. pneumoniae* ATCC 13883, *E. coli* ATCC 25922, *E. cloacae* ATCC 23355, *P. vulgaris* ATCC 13315, *P. aeruginosa* ATCC 27853, and 2 clinical isolates of *Mycobacterium* other than tuberculosis (Fig. 2).

DISCUSSION

Young *et al.* (1985) constructed recombinant λgt11 from randomly sheared chromosomal DNA of *M. tuberculosis* Erdman TMC 107, lot 9A-2 and screened by using monoclonal antibody. In this study, DNA fragments of *M. tuberculosis* H37Rv ATCC 27294, fully restricted with EcoRI, were used for the library construction. So DNA genes containing EcoRI restriction site were not included in the expression library of this study.

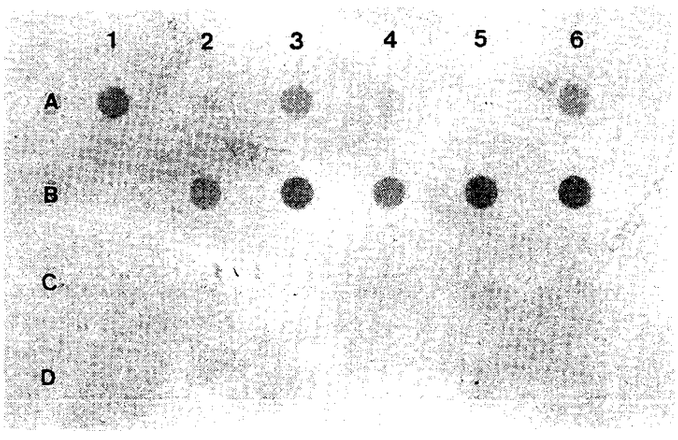

Fig. 2. Dot blot hybridization with digoxigenin-labeled 1.5-kb probe. A1, *M. tuberculosis* H37Rv; A2, *M. scrofulaceum*; A3, *M. bovis*; A4, *M. intracellulare*; A5, *M. tuberculosis* H37 Ra; A6, *M. tuberculosis* H37Rv; B1 & C6, 2 clinical isolates of *Mycobacterium* other than tuberculosis; B2-B6, 5 clinical isolates of *M. tuberculosis*; C1, *S. aureus*; C2, *S. epidermidis*; C3, *E. col*; C4, *S. marcescens*; C5, *K. pneumoniae*; D1, *E. cloacae*; D2, *P. vulgaris*; D3, *P. aeruginosa*

Mouse anti-*Mycobacterium tuberculosis* monoclonal antibody 5G2 (Chemicon, cat. No. MAB 848) showed no positive plaque forming unit in the screening of recombinant λgt11 library(data not shown). This result could be explained by the gene product against monoclonal antibody containing EcoRI restriction site or no reactivity of monoclonal antibody to *M. tuberculosis* H37Rv ATCC 27294.

Young *et al.* (1987) could obtain 29 positive clones from screening of 5 X 10^5 recombinant phages by rabbit polyclonal antibody against *M. tuberculosis* and reported that all of these clones were also reactive with anti-*M. bovis* BCG antibody (DAKO). This may be originated from the broad cross-reactivity between the two species. So the screening of the recombinant expression library was performed by using polyclonal antibody against *M. bovis* in this study (Harboe *et al.* 1979). *Mycobacterium tuberculosis* complex consists of *M. tuberculosis, M. africanum, M. microti and M. bovis*. DNA probe selected by polyclonal antibody screening can identify *M. tuberculosis* complex but cannot discriminate the exact species level. The probability that an isolate is *M. bovis* is quite low in Korea, and this organism is readily identified in probe-positive isolates by negative niacin test. The other members of the *M. tuberculosis* complex would be extremely rare: *M. africanum*, found in tropical Africa, is probably not a distinct species; and *M. microti*, occurring in voles and other animals, may be regarded as a biovar of *M. tuberculosis* (Ellner and Kiehn 1989).

Eisenach *et al.* (1986) could not find any difference on the restriction enzyme patterns between *M. tuberculosis* and *M. bovis*, and

reported 4 clones, obtained from BamHI-restricted λ1059 library, which reacted only to *M. tuberculosis* H37Rv. Also they isolated 3 clones from MboI-restricted M13 library, which exist repeatedly 10 - 16 times on the chromosomal DNA (Eisenach *et al.* 1988)

1.5-kb insert DNA obtained directly from EcoRI-restricted liquid lysate DNA of recombinant λgt11 clone showed a positive reaction with *M. tuberculosis* but also with E. coli *(data not shown). This might be derived from the contamination of E. coli* DNA in the liquid lysate. Therefore, 1.5-kb insert DNA was recloned into pBluescript plasmid for the amplification. Recombinant pBluescript ligated with 5-kb DNA(pBSTB) was 4.4 kb in size, which was confirmed by the digestion with Hind and BamHI. pBSTB labed with digoxigenin-dUTP was hybridized with DNA of *M. tuberculosis, E. coli, S. marcescens, K. pneumoniae* and *P. vulgaris*(data not shown). Labeling of total pBSTB increased the detection sensitivity to 1 ng of DNA, but could not be used as DNA probe for mycobacterial identification due to its nonspecific hybridization.

One of the problems in the application of the probe for the direct detection from sputum specimens is low sensitivities of this dot blot system. This technique can detect more than 2 X 10^7 mycobacteria, which corresponds to the number of organisms in sputums with 3^+ or 4^+ according to the report grading system for sputum smears of the Centers for Disease Control, Atlanta, Georgia. Sputums with 1^+ or 2^+ acid-fast stain could not be directly detected by this system without the mycobacterial culture. Therefore, this dot-blotting system cannot be recommended for the direct detection of *M. tuberculosis* from sputum.

Since 1.5-kb probe did not hybridize with avirulent strain of *M. tuberculosis* H37Ra ATCC 25177, the 1.5-kb gene may be associated with the virulence of *M. tuberculosis*. This suggestion should be clarified through further studies.

Whatever the function of the 1.5-kb gene selected in *M. tuberculosis* might be, this dot blot hybridization technique using digoxigenin-labeled DNA probe may be useful for the rapid identification of the *M. tuberculosis* complex in hospital laboratories.

ACKNOWLEDGEMENT

I would like to thank Young Soon Youn for her technical assistant.

REFERENCES

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. Current protocols in molecular biology. John Wiley & Sons, New York, 1987

Baess I. Determination and re-examination of genome sizes and base ratios on deoxyribonucleic acid from mycobacteria. Acta Path Microbiol Immunol Scand Sect B 1984;92:209-11

Collins DM, De Lisle GW. DNA restriction endonuclease analysis of *Mycobacterium tuberculosis* and *Mycobacterium bovis BCG. J Gen Microbiol 1984;130:1019-21*

Eisenach KD, Crawford JT, Bates JH. Genetic relatedness among strains of the Mycobacterium tuberculosis comlex-Analysis of restriction fragment heterogeneity using cloned DNA probes. Am Rev Respir Dis 1986;133:1065-8

Eisenach KD, Crawford JT, Bates JH. Repetitive DNA sequences as probes for *Mycobacterium tuberculosis*. J Clin Microbiol 1988;26:2240-5

Ellner PD, Kiehn TE. Author's reply. In Heifets L. Letter to the editor. Gen-Probe test should not be considered final in *Mycobacterium tuberculosis identification*. J Clin Microbiol 1989;27:229

Harboe M, Mshana RN, Closs O, Kronvall G, Axelsen NH. Cross-reactions between mycobacteria II. Crossed immunoelectrophoretic analysis of soluble antigens of BCG and comparison with other mycobacteria. Scand J Immunol 1979;9:115-24

Huynh TV, Young RA, Davis RW. Constructing and screening cDNA libraries in gt10 and

gt11. In Glover DM (Ed) DNA cloning. A practical approach. Vol I,IRL press, Oxford Washington DC, 1985: pp. 49-78

Roberts GD, Koneman EW, Kim YK. Mycobacterium. In Balows A, Hausler WJ, Herrman KL, Isenberg HD, Shadomy HJ(Ed) Manual of clinical microbiology, American Society for Microbiology, Washington,D. C. 1991: pp. 304-39

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, New York, 1989

Shoemaker SA, Fisher JH, Scoggin CH. Techniques of DNA hybridization detect small numbers of mycobacteria with no cross-hybridization with nonmycobacterial respiratory organisms. Am Rev Respir Dis 1985;131: 760-3

Young DB, Kent L, Young RA. Screening of a recombinant mycobacterial DNA library with polyclonal antiserum and molecular weight analysis of expressed antigens. Infect Immun 1987;55:1421-5

Young RA, Bloom BR, Grosskinsky CM, Ivanyi J, Thomas, Davis RW. Dissection of Mycobacterium tuberculosis antigens using recombinant DNA. Proc Natl Acad Sci USA 1985;82:2583-7